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6-Aminonicotinamide (6AN) has been shown to enhance the effect of radiation both in vivo and in vitro in a murine tumor models. This study was undertaken to determine whether enhanced efficacy of radiation, adriamycin and taxol could be obtained by pretreating with 6AN. MCF-7 cells were grown on beads and perfused in the NMR magnet. P31 NMR spectra were obtained on cells at 4.7T (81.03 MHz) which were treated with 6AN or control. Treatment with 6AN caused a new peak to be detected which has been previously assigned to 6-phosphogluconate. In addition, a decrease in phosphocreatine was noted. These results were analogous to results found with murine RIF-1 tumor cells. Surviving fraction studies were subsequently done. Cells were perfused for 4 hours with 6AN, the 6AN washed out and the cells incubated for 3 hours before being treated with radiation, adriamycin or taxol. Enhanced efficacy to radiation (2 Gy dose) and adriamycin (10 nM) was noted. 6AN inhibited the effect of Taxol. In vivo studies have been started and it appears that the maximum tolerated dose for 6AN will be between 10 and 12 mg/kg

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## Introduction

The focus of this research was to evaluate the potential of 6-Aminonicotinamide (6AN) to enhance response of breast tumors to chemotherapy (adriamycin and paclitaxel) and/or radiation (XRT). The aims of the overall project are to test 6AN, both in vivo and in vitro, on two tumor models, a hormone resistant and hormone sensitive model (MCF-7). The work to date has focused on in vitro studies of the MCF-7 and preliminary data (dose testing) has been obtained in vivo.

## Body

Methods: Cell culture. MCF-7 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Essential Medium (DME) and Minimal Essential Medium (MEM) mixed 1:1 and supplemented with 5% fetal bovine serum, 100U/ml penicillin-G and 100ug/ml Streptomycin, 1mM pyruvate and 0.25 U/ml bovine insulin (Intergen, Purchase, NY). The concentration of glucose in the medium was 15 mM. MEM was supplemented with non-essential amino acids and the final concentration of each was 0.5mM. For NMR studies, cells were grown on Cultispher gelatin beads (HyClone Laboratories, Inc., Logan, UT) in the same medium described above except that it was supplemented with 10% FBS. Cells ( $1 - 2 \times 10^7$ ) were mixed with 0.2 g beads in 50ml medium inside a spinner flask. Cells and beads were left 18 - 24 hours without stirring. Subsequently, cells and beads were stirred at 30 - 40 rpm. Cells were cultured on the beads for 5 - 7days before being used for NMR studies. Readiness of cells was judged visually by mixing a small amount of cells with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma) which is taken up by cells and converted to an insoluble, purple product by dehydrogenases. After 30 minutes beads, were inspected under a microscope to see the extent of cell confluence on the beads. Experiments were started if greater than 90% of the beads were greater than 50% covered with cells.

For cytotoxicity studies,  $1.3 \times 10^5$  cells were subcultured in T-25 flasks. Four days later cells were exposed to 6-AN for 4 hours. Media containing 6-AN was replaced with fresh media and 3 hours later cells were exposed to radiation, adriamycin or taxol. In the case of adriamycin, cells were exposed for 4 hours; in the case of taxol, cells were exposed for 24 hours. At the end of adriamycin and taxol exposures, cells were trypsinized and counted using a Coulter Counter. After radiation, cells were returned to the incubator for 3 hours of recovery and then trypsinized and counted. Cells (400 - 2000 cells) were plated into 6-well plates containing 2 ml media described above but supplemented with 5 or 10% FBS and 1U/ml insulin. After 2 - 3 weeks, clones of over 100 cells were counted by staining with 1% crystal violet in absolute methanol.

NMR measurements. MCF-7 cells grown on collagen beads were placed into a 10mm (or 12mm) shortened, screw-cap NMR tube which was connected to a perfusate reservoir via tubing inserted through a teflon/silicone septum (1,2). Inlet tubing carried perfusate from the 500 ml reservoir-bottle to the bottom of the NMR tube. The pumping speed was adjusted so that the beads floated to the beginning of the widest cross-sectional area of the tube, about a centimeter from the top. Outlet tubing carried perfusate from the top of the NMR tube, above the top of the beads, back to the reservoir bottle. The outlet tubing ended with a T-connection that allowed switching between the perfusate-reservoir (closed system) and a waste-reservoir (open system). Switching to an open system allowed washout of drug. The perfusate consisted of phosphate-free DME

supplemented with 10% FBS, 1mM pyruvate and 0.25U/ml insulin. Oxygenation of the perfusion media was accomplished by blowing O<sub>2</sub>/CO<sub>2</sub> (95%:5%) over the top of the media in the reservoir.

NMR spectra of phosphorus metabolites were collected using a five-turn solenoid coil that was wrapped around the NMR tube (1,2). WALTZ decoupled <sup>31</sup>P spectra were obtained using a Bruker/GE 4.7 T Omega spectrometer (Bruker NMR, Fremont, CA) operating at 81.03 MHz. Data was acquired with a spectral width of 10,000 Hz, a 60° pulse angle, a recycle time of 2 seconds, 2048k data points and 2048 signal averaged free-induction decays (FID). To quantify metabolites, FID's of 2 sequential acquisitions were summed to improve signal-to-noise and the areas under the curve for metabolites of interest were estimated using Magnetic Resonance User Interface software package (MRUI). The AMARES fitting routine was employed to fit a Lorentzian function to the time domain signal. Spectra were processed with 5 Hz exponential line broadening (3).

## Results

Task 1 – The perfusion scheme for MCF-7 cells was validated. The perfused cells (MCF-7) were metabolically stable for 48 hours when perfused with phosphate-free DME (data not shown). A higher signal-to-noise ratio was achieved with cells grown on beads using the petri dish method than with cells grown on beads in a spinner flask. This was probably due to greater cell density as indicated by the qualitative observation that beads incubated with cells in a petri dish had greater MTT staining than beads incubated with cells in a spinner flask. NMR coils have been constructed both for in vivo and in vitro studies.

Task 3 – The effect of 6AN on tumor metabolism has been studied (MCF-7). Spectra have been obtained both without (data not shown) and with 6AN (see figure 1, Appendix 1). 6-phosphogluconate (6-PG) levels rose from undetectable in the baseline spectrum to become the largest peak in the spectrum. Quantitative assessment of the effect of 6-AN on 6-PG/Pi, and PCr/Pi is demonstrated in Figure 1. 6-PG/Pi rose to a maximum level by 10 hours after the end of the 6-AN perfusion. There was no change in βNTP levels but PCr levels decreased indicating that energy metabolism was inhibited. (see Fig. 1, appendix 1, and Fig. 1)

Task 4 – A 4 hour exposure to 6-AN (dose range studied 0-200μM) did not effect plating efficiency of MCF-7 cells. However, at 80 and 200 μM, 6-AN decreased the surviving fraction of cells exposed to 2 Gy (Fig. 2). Exposure to radiation before exposure to 6-AN abolished the effect of 6-AN on MCF-7 cell radiosensitivity (Figure 3) indicating that metabolism of 6-AN is necessary to cause radiosensitization.

6-AN increased the effectiveness of 10<sup>-8</sup> M adriamycin to decrease MCF-7 cell clonogenicity (p<0.05) but had no effect when adriamycin was present at higher levels. This finding may indicate that the two drugs have in common cellular processes or targets which are damaged or changed and through which the drugs exert their cytotoxic effects. At low levels of adriamycin these process or targets are not completely saturated and thus the addition of 6-AN may cause an increase in cytotoxicity. At higher levels of adriamycin, there is significant cell kill and therefore the apparent potentiation by 6-AN disappears.

In contrast to the results of the adriamycin experiments, 6-AN appears to inhibit the cytotoxicity of paclitaxel. This effect is observed over all of the paclitaxel levels studied (Figure

4) and indicates that 6-AN may inhibit cell cycle kinetics as paclitaxel is toxic to cells in mitosis. Studies are ongoing to determine the effect of 6AN on cell cycle distribution by flow cytometry.

Task 5 – Tumor implantation (MCF-7) has been started for measuring tumor doubling times and the effect of 6AN on enhancing tumor response.

It is obvious that we have decided to do the studies of the tumor models sequentially instead of in parallel. The PI was forced to change plans since Dr. Holleran left the laboratory for a better position. Since she performed the in vitro studies, it was elected to move to the in vivo studies while a replacement is recruited. Thus we have completed about 1/3 of the grant, but not in the order initially planned in the statement of work.

### Figure Legends

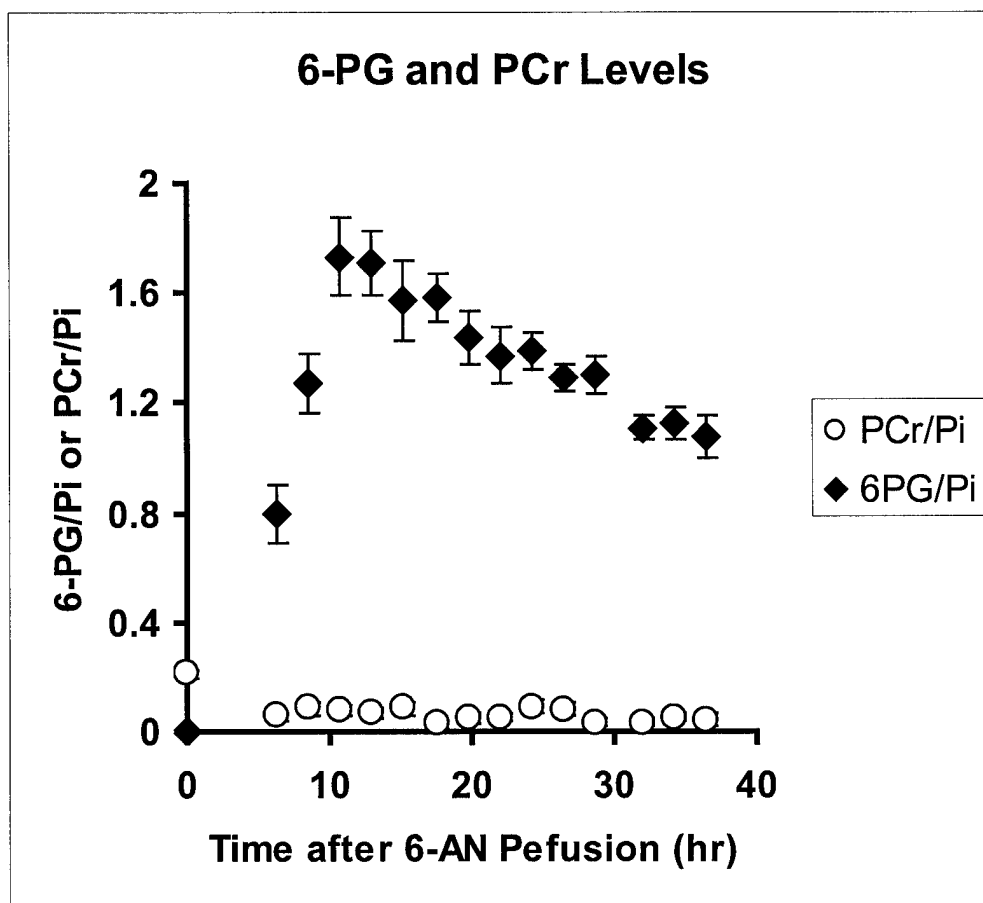
Figure 1. Changes in 6PG/Pi and PCr/Pi after treatment with 200uM 6AN. By seven hours after beginning exposure to 6AN, there are significant metabolic effects noted.

Figure 2. Surviving fraction measurements after exposure to 6AN and 2 Gy of radiation demonstrating significant decreases in survival of human MCF-7 cells after the combination of 6AN→XRT.

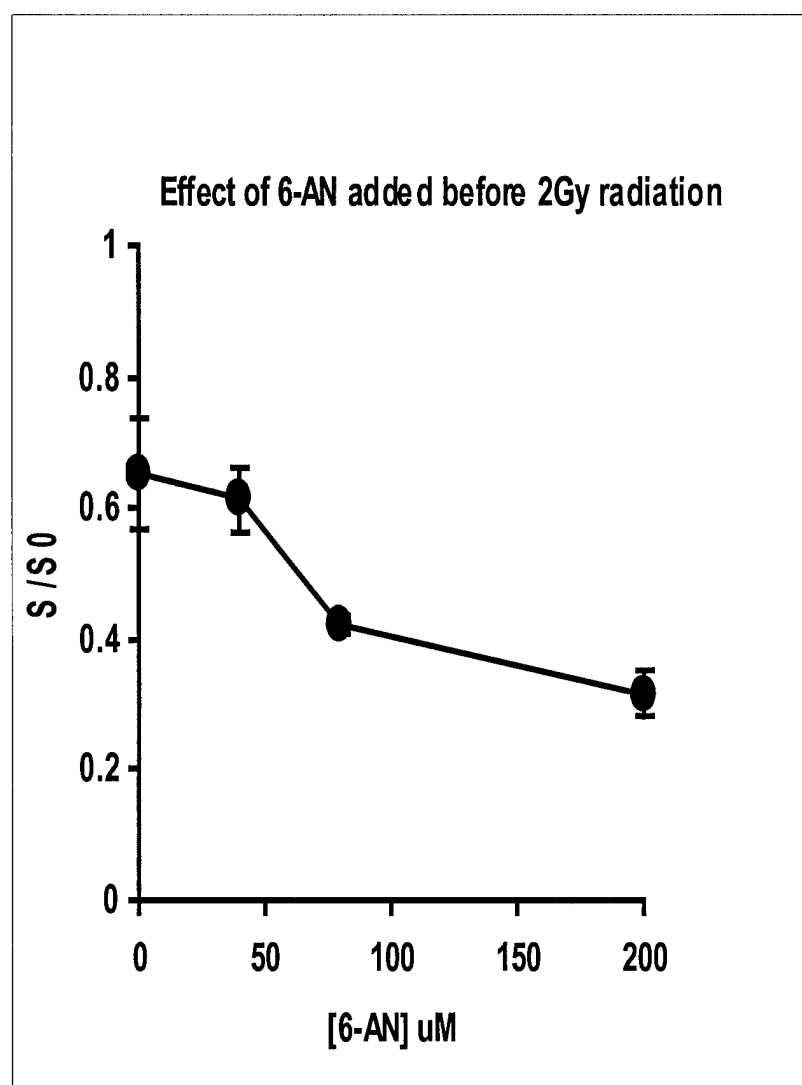
Figure 3. Surviving fraction measurements after exposure to 2 Gy of radiation followed by 6AN. Data demonstrate no effect when XRT is administered before 6AN.

Figure 4. Surviving fraction measurements after exposure to 6AN and taxol. 6AN inhibits the effect of taxol.

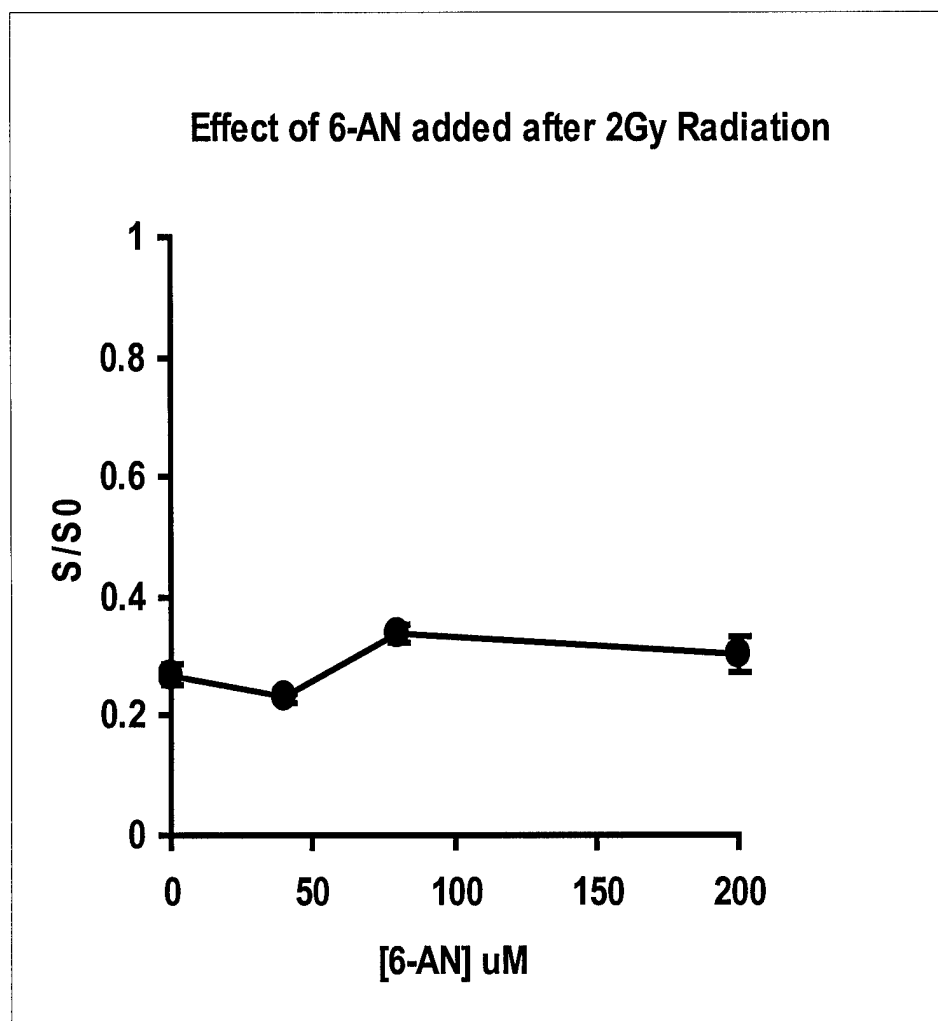




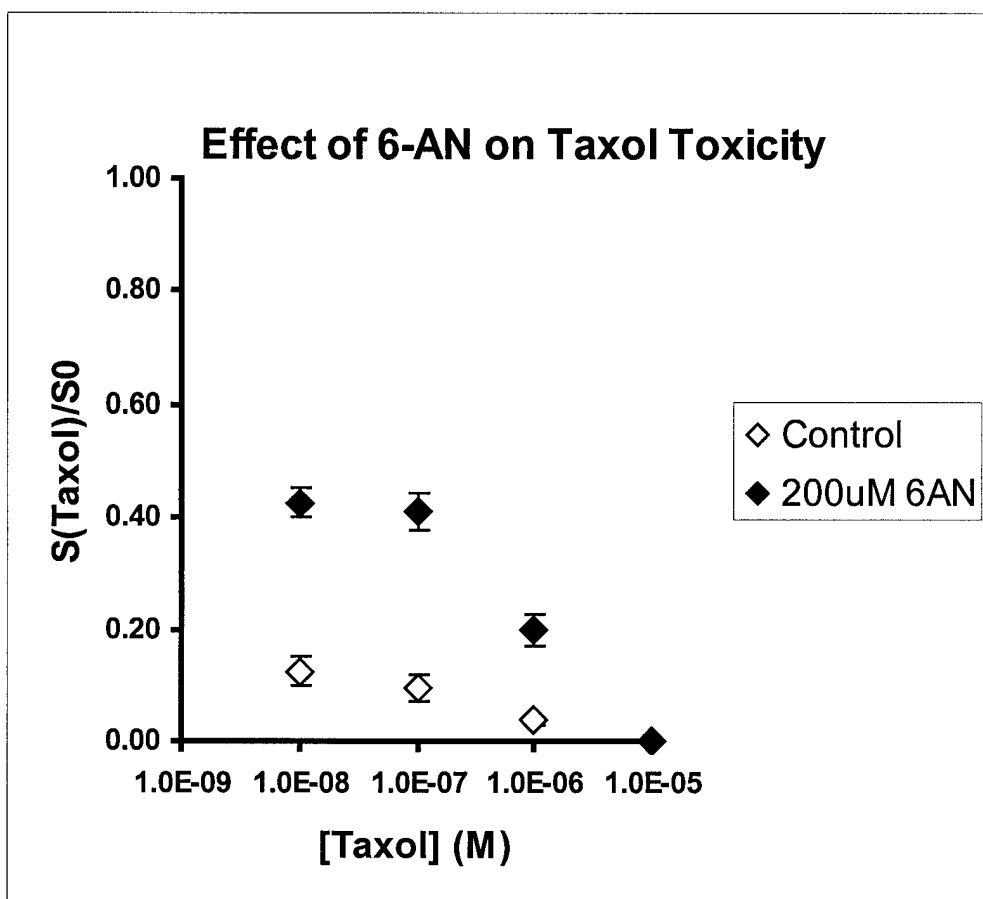
**Figure 1**



**Figure 2**



**Figure 3**

**Figure 4**

## Key Research Accomplishments

1. Enhancement of cytotoxicity of radiation by 6AN.
2. Enhancement of cytotoxicity of adriamycin by 6AN
3. Lack of enhancement of cytotoxicity of taxol by 6AN. 6AN appears to interfere with the efficacy of 6AN.

## Reportable Outcomes

1. Holleran A, Chen, Y and Koutcher JA. Effect of 6-Aminonicotinamide on Human Tumor Metabolism, and Response to Chemotherapy and Radiation. Int Soc Magnetic Resonance in Medicine, 7<sup>th</sup> Ann. Mtg. Philadelphia, Pa. May 23-29<sup>th</sup>, 1999.

## Conclusions

1. 6AN is an effective sensitizer in vitro for both radiation and adriamycin. It may inhibit the efficacy of taxol in vitro and further studies are necessary. In vivo studies to determine the efficacy of 6AN in vivo have been started. If the in vivo results are also positive, a study in patients could be contemplated. This would likely require preclinical studies to determine the efficacy of 6AN on a daily basis for radiation enhancement prior to clinical studies. Since adriamycin is given once over a 1-3 week interval, further preclinical studies may not be necessary.
2. An unexpected finding, is that the nude mice are more sensitive to 6AN than C3H and CD8F1 mice. The maximum tolerated dose (MTD) for the latter mice is 20mg/kg administered every 10-11 days. It appears that the MTD for nude mice may be ~ 10-12mg/kg. This is being evaluated.
3. The unexpected effect of 6AN on taxol, has prompted us to do so cell cycle studies to determine if 6AN effects cell cycle distribution which could interfere with taxol's efficacy.

## References

1. Street JC, Mahmood U, Ballon D, Alfieri A, and Koutcher JA. <sup>13</sup>C and <sup>31</sup>P NMR investigation of effect of 6-Aminonicotinamide on metabolism of RIF-1 tumour cells in vitro. J. of Biol. Chem. 271:4113-4119, 1996.
2. Street JC, Alfieri AA, and Koutcher JA. Quantitation of metabolic and radiobiological effects of 6-Aminonicotinamide in RIF-1 tumor cells in vitro. Cancer Res. 57: 3956-3962, 1997.
3. van der Veen JWC, de Beer R, Luyten PR and van Ormondt D. Accurate quantification of in vivo <sup>31</sup>P NMR signals using the variable projection method and prior knowledge. Magnetic Resonance Med, 6: 92-98, 1988.

## Appendices

Appendix 1 - attached

# Effect of 6-Aminonicotinamide on Human Tumor Metabolism, and Response to Chemotherapy and Radiation

Anne Holleran\*, Yuchun Chen, and Jason A. Koutcher  
US Patent Office\* and Memorial Sloan Kettering Cancer Center

## Introduction

Previous studies from different investigators (1,2) have indicated that 6-aminonicotinamide (6AN) can enhance anti-neoplastic treatment. In a previous study of RIF-1 cells, the surviving fraction after radiation was decreased if the cells were pretreated with 6AN (1). We have investigated the effect of 6AN on survival of human mammary tumor cells (MCF-7) after treatment with radiation, adriamycin and paclitaxel.

## Methods

**Cells – NMR –** MCF-7 cells were grown on Culti-sphere gelatin beads in media consisting of 50% DMEM and 50% minimal essential media with 10% FBS. Cells ( $1-2 \times 10^7$ ) were mixed with 0.2 g beads in 50 ml media in a spinner flask for 18-24 hours without stirring. They were subsequently stirred for about 4-6 days at 30-40 rpm. Experiments were started when greater than 90% of the beads were greater than 50% covered with cells.

**Cells – cytotoxicity studies –**  $1.3 \times 10^5$  cells were subcultured for 4 days and subsequently exposed to 6AN for 4 hours. 6AN containing media was replaced with fresh media and 3 hours later the cells were exposed to radiation (2,3, or 4 Gy), adriamycin (4 hours) or paclitaxel (24 hours). Subsequently, the cells were trypsinized and counted using a Coulter Counter. After radiation, cells were returned to the incubator for 3 hours of recovery and then trypsinized and counted.

**NMR –** MCF-7 cells grown on collagen beads were placed in a 12 mm shortened screw cap NMR tube which was connected to a perfusate reservoir via tubing. The pumping speed was adjusted so that the beads floated to the beginning of the widest cross-sectional area of the tube, about a cm from the top. NMR spectra were collected using a 5 turn solenoid coil wrapped around the 12 mm NMR tube. WALTZ decoupled  $^{31}\text{P}$  spectra were obtained using a 4.7T Bruker Omega CSI system operating at 81.03 MHz. Spectra were obtained prior to perfusion with 6AN, during the 4 hours of 6AN exposure (200uM) and subsequently after washout of 6AN for about 24 hours. Each spectrum required about 70 minutes to acquire.

## Results

Figure 1 shows a series of spectra obtained on perfused MCF-7 cells. The peaks detected prior to 6AN perfusion include phosphoethanolamine (PE) (B), phosphocholine (PC) (C), inorganic phosphate (Pi) (D), glycerophosphoethanolamine (GPC) (E), glycerophosphocholine (GPC) (F), phosphocreatine (PCr) (G),  $\gamma$ ,  $\alpha$  and  $\beta$  NTP (peaks H, I and L), and diphosphodiester (DPDE) (peaks J and K). The Pi peak was usually split into two components after treatment with 6AN which likely represented resolution of intra- and extra-cellular pH. During the infusion of 6AN and subsequently, a peak previously assigned to 6-phosphogluconate (6PG) was detected (A). As in studies with the RIF-1, this was the dominant peak in the spectrum and was visible for >24 hours post washout of 6AN. The baseline spectrum in Fig. 1 required 70 minutes while subsequent spectra required 135 minutes of data acquisition.

The effect of 4 Gy of radiation was significantly enhanced by pretreatment with 6AN. The surviving fraction (SF) after 4 Gy was  $0.087 \pm 0.007$  compared to  $0.036 \pm 0.018$  for cells exposed to 6AN before radiation. 6AN alone had no effect on

SF; similarly radiation followed by 6AN did not show an enhanced effect.

Pretreatment with 6AN (200uM) resulted in an enhanced efficacy of adriamycin at low concentrations ( $10^{-8}$  M), but this effect was lost at higher concentrations. This may be due to the very low cell survival that was present at higher doses of adriamycin. Pretreatment with 6AN followed by paclitaxel failed to show any enhancement. The activity of paclitaxel is dependent on cells traversing mitosis and therefore further studies to determine if 6AN may inhibit cells from entering mitosis are ongoing.

The current study corroborates a previous study (1) which showed enhancement of the effect of radiation by 6AN. The previous study was done in a murine tumor cell line, whereas this study demonstrates radiation enhancement in a human tumor cell line. Furthermore, this study also shows enhancement with adriamycin, one of the two most active agents used in breast cancer. Since adriamycin has a risk of cardiac toxicity at higher doses, it may be feasible by adding 6AN to achieve equi-efficacious activity with a lower dose of adriamycin, thereby decreasing the risk of cardiac toxicity.

## References

1. Street et al J. Biol. Chem, 271, 4113, 1996
2. Budihardjo II et al Clin Cancer Res. 117, 1998

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